THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 21, Issue of May 23, pp. 19280–19285, 2003
Printed in U.S.A.

Microarray Analysis Reveals Interleukin-6 as a Novel Secretory Product of the Hypothalamo-neurohypophyseal System*

Received for publication, September 26, 2002, and in revised form, March 3, 2003
Published, JBC Papers in Press, March 14, 2003, DOI 10.1074/jbc.M209902200

Mohamed T. Ghorbel‡§, Greig Sharman‡¶, Marie Leroux‡, Tanya Barrett‖, David M. Donovan, Kevin G. Becker‖, and David Murphy‡**

*From the ‡University Research Centre for Neuroendocrinology, University of Bristol, Bristol Royal Infirmary, Marlborough Street, Bristol BS2 8HW, United Kingdom, the ‖DNA Array Unit, NIA, National Institutes of Health, Baltimore, Maryland 21224, and the **Biotechnology and Germplasm Laboratory, United States Department of Agriculture, Beltsville, Maryland 20705*

Physiological activation of the hypothalamo-neurohypophyseal system (HNS) by dehydration results is a massive release of vasopressin (VP) from the posterior pituitary. This is accompanied by a functional remodeling of the HNS. In this study we used cDNA arrays in an attempt to identify genes that exhibit differential expression in the hypothalamus following dehydration. Our study revealed nine candidate genes, including interleukin-6 (IL-6) as a putative novel secretory product of HNS worthy of further analysis. *In situ* hybridization and immunocytochemistry confirmed that IL-6 is robustly expressed in the supraoptic (SON) and the paraventricular (PVN) nuclei of the hypothalamus. By double-staining immunofluorescence we showed that IL-6 is largely co-localized with VP in the SON and PVN. *In situ* hybridization, immunocytochemistry, and Western blotting all revealed IL-6 up-regulation in the SON and PVN following dehydration, thus validating the array data. The same dehydration stimulus resulted in an increase in IL-6 immunoreactivity in the axons of the internal zone of the median eminence and a marked reduction in IL-6-like material in the posterior pituitary gland. We thus suggest that IL-6 takes the same secretory pathway as VP and is secreted from the posterior pituitary following a physiological stimulus.

The hypothalamo-neurohypophyseal system (HNS)[1] consists of large magnocellular neurons (MCNs) of the supraoptic (SON) and paraventricular (PVN) hypothalamic nuclei that have axons terminating on blood capillaries of the posterior pituitary (1), along with associated glia, blood vessels, interneurons, and afferent terminals. The HNS is the source of the neuropeptide hormone vasopressin (VP), which has a crucial role in osmoregulation (2). Following the onset of an osmotic stimulus such as dehydration (fluid deprivation), mammals respond to plasma hyperosmolality by reducing the renal excretion of water. Tubular reabsorption of water is controlled by circulating levels of VP. A rise in plasma osmolality is detected by osmoreceptor mechanisms in the circumventricular organs (3–6). Subsequent angiotensinergic (7, 8) and glutamatergic (9–11) excitation of hypothalamic neurons leads to a massive release of stored VP into the general circulation. Through an interaction with V2-type receptors located in the kidney, VP increases the permeability of the collecting ducts to water, promoting water conservation by decreasing the amount of water lost in urine.

Physiological activation of the MCNs by dehydration results in a massive release of stored hormone from posterior pituitary terminals, and a concomitant functional remodeling of the HNS, characterized by activity-dependent secretory, electrophysiological, biosynthetic, and gene expression plasticity (12–14). At the morphological level, this plasticity is manifested as synaptic remodeling, increased direct neuronal membrane apposition and dendritic bundling in the SON, and by changes in the organization of neurovascular contacts in the neurohypophysis (12). For example, alterations in the relationship between MCNs and glia, the extent of terminal contact with the basal lamina in the neurohypophysis, and the extent of electrotonic coupling between MCNs have all been described (12, 15, 16). However, the mechanistic basis of these effects has not yet been described in terms of the differential expression of genes.

We have used cDNA microarray gene expression profiling technology (17–19) to identify candidate genes that are differentially expressed in the SON following 3 days of dehydration. One of these genes, interleukin-6 (IL-6), has been reported to be involved in the hypothalamic-pituitary-adrenal axis activation (20–22) and therefore was studied in detail. We demonstrate robust expression of IL-6 mRNA and peptide in the SON and PVN, with significant up-regulation following dehydration. The concomitant decrease in IL-6-like immunoreactive material in the posterior pituitary suggests, for the first time, that this cytokine is a secretory product of the HNS.

EXPERIMENTAL PROCEDURES

Animals—Adult male Sprague-Dawley rats 10–12 weeks old were maintained in standardized conditions in accord with United Kingdom Home Office regulations. Dehydration involved complete fluid deprivation for 3 days.

Total RNA Preparation—Each experimental group was compared as replicates of three. For each replicate SONs from 12 rats were pooled before RNA extraction. Animals were sacrificed by cervical dislocation, and brains were removed. SONs were isolated on a coronal slice of 1–2 mm thick and then micro-dissected under microscope. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol.

* This work was supported by The Wellcome Trust research program (United Kingdom) (to M. T. G. and D. M.) and the NIA intramural research program (to K. G. B. and T. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Present address: MRC Centre for Synaptic Plasticity, Dept. of Anatomy, School of Medical Sciences, University Walk, University of Bristol, Bristol BS8 1TD, UK. Tel.: 44-117-954-6477; Fax: 44-117-929-1687; E-mail: m.ghorbel@bris.ac.uk.

¶ Recipient of scholarship from the Medical Research Council (United Kingdom).

[1] The abbreviations used are: HNS, hypothalamo-neurohypophyseal system; MCN, magnocellular neuron; SON, supraoptic nucleus; PVN, paraventricular nucleus; VP, vasopressin; IL, interleukin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; ISH, *in situ* hybridization; ME, median eminence.

Microarray Target Labeling and Hybridization—Targets for cDNA microarrays were generated using 5 μg of total RNA from control and dehydrated rat SON in a standard reverse transcription reaction. RNA was annealed, in 16 μl of water, with 1 μg of 24-mer poly(dT) primer (Invitrogen), by heating at 65 °C for 10 min and cooling on ice for 2 min. The reverse transcription reaction was performed by adding 8 μl of 5× first strand reverse transcription buffer (Invitrogen), 4 μl of 20 mM dNTPs minus dCTP (Amersham Biosciences), 4 μl of 0.1 M dithiothreitol, 40 units of RNase OUT (Invitrogen), 6 μl of 3000 Ci/mmol [^{33}P]dCTP (ICN Biomedicals) to the RNA/primer mixture to a final volume of 40 μl. Two μl (400 units) of Superscript II reverse transcriptase (Invitrogen) was then added, and the sample was incubated for 30 min at 42 °C followed by additional 2 μl of Superscript II reverse transcriptase and another 30-min incubation. The reaction was stopped by the addition of 5 μl of 0.5 M EDTA. The samples were incubated at 65 °C for 30 min after addition of 10 μl of 0.1 M NaOH to hydrolyze and remove RNA. The samples were pH neutralized by the addition of 45 μl of 0.5 M Tris, pH 8.0, and purified using Bio-Rad 6 purification columns (Bio-Rad). The NIA neuroarray consists of 1152 cDNAs printed on nylon membrane in duplicate (23). The arrays were hybridized with [α-^{33}P]dCTP-labeled cDNA probes overnight at 50 °C in 4 ml of hybridization solution. Hybridized arrays were rinsed in 50 ml of 2× SSC and 1% SDS twice at 55 °C followed by washing one to two times with 2× SSC and 1% SDS at 55 °C for 15 min each. The microarrays were exposed to PhosphorImager screens for 1–3 days. The screens were then scanned with Amersham Biosciences STORM PhosphorImager (Sunnyvale, CA) at 50-μm resolution.

Microarray Data Analysis: z Normalization—ImageQuant software (Amersham Biosciences) was used to convert the hybridization signals on the image into raw intensity values, and the data thus generated were transferred into MS Excel spreadsheets, predesigned to associate the ImageQuant data format to the correct gene identities. Raw intensity data for each experiment was normalized by *z* transformation. Intensity data were first, log10-transformed and used for the calculation of *z* scores. *z* scores were calculated by subtracting the average gene intensity from the raw intensity data for each gene and dividing that result by the S.D. of all the measured intensities. Gene expression differences between any two experiments were calculated by taking the difference between the observed gene *z* scores. The significance of calculated *z* differences can be directly inferred from measurements of the S.D. of the overall *z* difference distribution. Assuming a normal distribution profile, *z* differences are assigned significance according to their relation to the calculated standard deviation of all the *z* differences in any one comparison. To facilitate comparison of *z* differences between several different experiments, *z* differences were divided by the appropriate standard deviation to give the *z* ratios (23).

Immunocytochemistry—Deeply anesthetized rats were perfused with 60 ml PBS and then with 60 ml of 4% (w/v) paraformaldehyde. Brains were then dissected into 4% (w/v) paraformaldehyde and postfixed overnight. Following overnight cryoprotection in 30% (w/v) sucrose in PBS for 1 day, 30-μm (for brains) and 16-μm (for pituitaries) cryostat (Leica Instruments, Japan) sections were collected into PBS for immunocytochemistry. Following two 10-min washes in PBS, sections were blocked with PBS containing 0.3% (v/v) Triton X-100 and 1% (w/v) bovine serum albumin (BSA). Primary antibodies (affinity-purified goat polyclonal antibody raised against a peptide mapping at the carboxyl terminus of the IL-6 precursor of rat origin (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse monoclonal anti-neurophysin-II antibody that label VP expressing cells, generously provided by Dr. J. F. Morris, University of Oxford, Oxford, UK) at 1:100 dilution were incubated overnight at 4 °C with the sections in PBS containing 0.3% (v/v) Triton X-100 and 1% (w/v) BSA. Following three 10-min washes with PBS at room temperature, an appropriate dilution (1:100) of fluorescent secondary antibody (FITC or Texas Red®, Sigma) in PBS containing 0.3% (v/v) Triton X-100 and 1% (w/v) BSA was added to the sections. Sections were incubated at room temperature for 2 h. After washing the sections in PBS in the dark, they were mounted in Vectashield (Vector Laboratories) and observed under a fluorescent microscope.

In Situ Hybridization (ISH)—A mixture of the two following oligonucleotides was used for making the IL-6 antisense probes: 5′-GGG AAG GCA GTG GCT GTC AAC AAC ATC AGT CCC-3′ (nt 141 to nt 109) and 5′-GAG TTG GAT GGT CTT GGT CCT TAG CCA CTC CTT CTG TGA CTC-3′ (nt 640 to nt 599).

Radioactive ISH—Brain tissue was flash-frozen on dry ice. 12-μm sections were prepared on Superfrost plus slides (BDH) and incubated with [α-^{35}S]dATP-end-labeled sense and antisense oligonucleotide probes (Promega, UK) as described previously (24). Slides were washed four times in 1× SSC at 55 °C for 15 min each time and twice in 1× SSC at room temperature for 30 min each time. The air-dried slides were exposed to Hyperfilm® (Amersham Biosciences) for 4 weeks before development. The exposed films were scanned and the optical densities were correlated to ^{14}C standards (Micro-scales, Amersham Biosciences) co-exposed to the film. Image analysis was carried out by NIH-Image software (National Institutes of Health, Bethesda, MD). Quantification was achieved using 24 sections from three different animals.

Non-radioactive ISH with Tyramide Signal Amplification—Oligonucleotides were 3′-end labeled with digoxygenin-11-dUTP using terminal transferase and standard protocol provided by Roche Applied Science (Basel, Switzerland). Sixteen-micron-thick sections were then placed in PBS prior to prehybridization at 42 °C for 2 h in 44% formamide, 2× SSC, 5× Denhardt's solution, 0.2% SDS, 50 mg/ml heparin, 25 mg/ml denatured salmon sperm DNA, 25 mg/ml yeast tRNA, 30% diethylpyrocarbonate-treated water, then immersed in hybridization buffer (44% formamide, 2× SSC, 5× Denhardt's solution, 0.2%SDS, 100 mg/ml heparin, 25 mg/ml denatured salmon sperm DNA, 25 mg/ml yeast tRNA, 30% diethylpyrocarbonate-treated water) containing 5 nM labeled oligonucleotide probe at 42 °C overnight. The sections were then washed twice 5 min at room temperature with 1× SSC, 0.1% SDS, once 5 min, then once 10 min at room temperature with 0.2× SSC, 0.1% SDS. Following post-hybridization washes, sections were washed in Tris-NaCl-Tween (TNT) buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20), blocked in TNB buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent) for 30 min at room temperature, then incubated in 1:100 anti-digoxygenin-horseradish peroxidase (Roche Applied Sciences) for 1 h at room temperature, washed in TNT buffer, then incubated in biotinyl tyramide (PerkinElmer Life Sciences) 1:50 for 10 min at room temperature. After washing in TNT buffer, section were incubated for 30 min in 1:500 streptavidin-horseradish peroxidase (PerkinElmer Life Sciences), which binds with high affinity to the locally deposited biotin labels. Probe labeling was visualized by diaminobenzidine reaction. Finally sections were mounted on glass slides, air-dried, and cover-slipped with Vectashield (Vector Laboratories) mounting medium.

Western Blot Analysis—Brain tissues were homogenized in 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM phenylmethylsulphonyl fluoride, 0.5 mM dithiothreitol, 25% (v/v) glycerol. The homogenate was then frozen on dry ice for 5 min, thawed in ice water for 15 min, and centrifuged at 14,000 × *g* for 15 min. The clear supernatant defined as total protein extract was removed and stored at −70 °C. Protein concentrations were determined using the Bradford method (Bio-Rad). Equal amounts of protein samples (30 μg) were separated on a 16% (w/v) SDS-polyacrylamide gel and electrotransferred onto Immobilon-P membrane (Millipore, Bedford, MA). Membrane preparations were blocked in 5% (w/v) BSA in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% (w/v) Tween 20) and then incubated overnight at 4 °C with affinity-purified goat polyclonal antibody raised against a peptide mapping at the carboxyl terminus of the murine IL-6 precursor (Santa Cruz Biotechnology) diluted 1:100 in TBS-T, 5% (w/v) BSA. Following four washes of 10 min each with TBS-T, membranes were incubated with a 1:100,000 dilution of horseradish peroxidase-coupled anti-goat (Sigma) secondary antibody for 1 h at room temperature, and bound antibody was visualized with the ECL chemiluminescence system (Amersham Biosciences). Membranes were opposed to x-ray films, which were developed according to the manufacturer's instructions. Films were scanned (Gel Doc 2000, Bio-Rad), and densitometric analysis was carried out using quantification software (QuantityOne, Version 4.2, Bio-Rad). Identical loading was checked by Coomassie Blue staining.

Serum IL-6 Measurements—Five rats per group were ensanguinated following dehydration for 0, 1, and 3 days. Serum samples were analyzed in duplicate for rat IL-6 using a specific enzyme-linked immunosorbent assay (Biotrak, Amersham Biosciences).

Statistics—The statistical analyses for both ISH and Western blotting were performed by one-way analysis of variance followed by Student's *t* test. Results were considered significant when *p* values were <0.05. Data analysis was performed using InStat program (InStat for Macintosh, Version 2.01).

RESULTS

Microarray Findings—NIA neuroarray probes were hybridized in triplicate with targets derived from SON of control rats and rats dehydrated for 3 days. Data analysis by *z* normalization of the hybridization signals enabled us to identify 9 candidate regulated genes. Four of these genes were up-regulated by dehydration, while five were down-regulated (Table I). Here, we pre-

TABLE I

Genes showing up- or down-regulation in the rat SON following 3 days of dehydration

The data represent array experiments carried out in triplicate for each experimental group. An independent RNA pool was used for each replicate. PI, phosphatidylinositol; EST, expressed sequence tag.

GenBank™ accession no.	UniGene Cluster ID	Gene name	Up/ Down	*z* ratio	S.E.
W99328	Hs.8121	ESTs, highly similar to notch homolog 2 (*Homo sapiens*)	Up	7.84	2.83
W30935	Hs.111460	ESTs, highly similar to multifunctional calcium/calmodulin-dependent protein kinase II Δ2 isoform (*H. sapiens*)	Up	4.05	1.35
N98591	Hs.93913	Interleukin 6 (interferon, β2; IL-6)	Up	2.6	0.44
H45000	Hs.74122	Caspase 4, apoptosis-related cysteine protease	Up	2.37	0.47
N78582	Hs.50732	Protein kinase, AMP-activated, β2 non-catalytic subunit	Down	−1.83	0.47
AA669443	Hs.334810	Eukaryotic translation initiation factor 5 (eIF-5)	Down	−1.93	0.22
AA464067	Hs.6453	Inositol 1,3,4-triphosphate 5/6 kinase	Down	−2.03	1.52
H98694	Hs.352382	PI 3-kinase-related kinase SMG-1	Down	−2.39	0.76
AA668470	Hs.24950	Regulator of G-protein signaling 5 (RGS5)	Down	−2.90	0.75

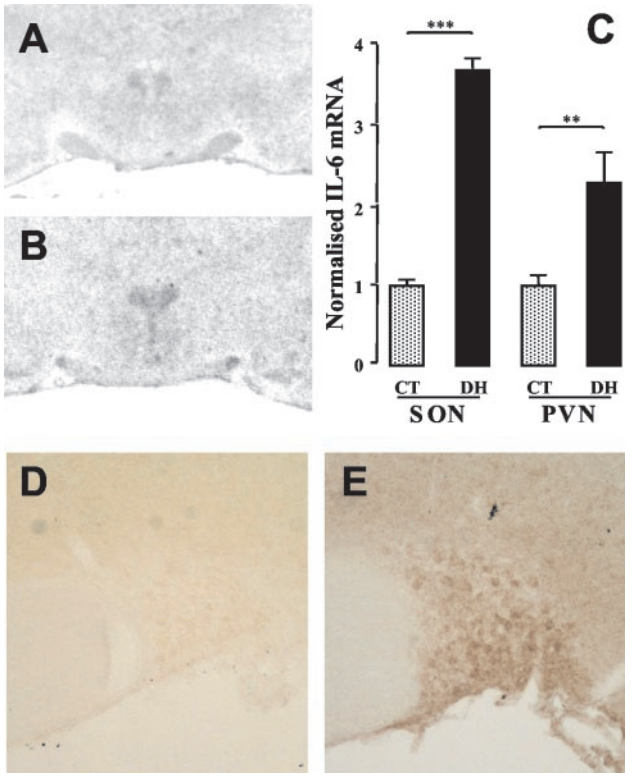

FIG. 1. **IL-6 *in situ* hybridization analysis.** Control (*A*) and dehydrated (*B*) rat brain sections were hybridized with an antisense oligo probe corresponding to rat IL-6 as described under "Experimental Procedures." Film autoradiographs were developed after 4 weeks then scanned. *C* represents a histogram of the normalized optical densities of IL-6 mRNA. *CT*, control; *DH*, dehydrated. $p < 0.05$ was considered significant. ***, $p < 0.001$; **, $p < 0.01$. *D* and *E* represent, respectively, non-radioactive ISH of control and dehydrated rat SON. Magnifications, ×8 (*A*, *B*) and ×80 (*D*, *E*).

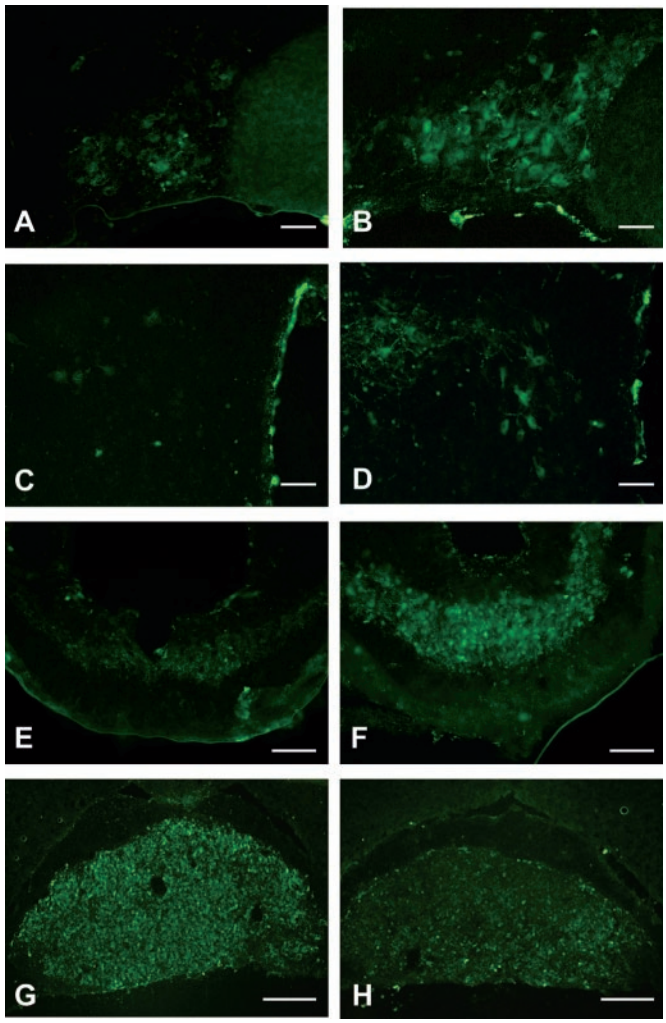

FIG. 2. **IL-6 immunoreactivity in the SON (*A*, *B*), PVN (*C*, *D*), ME (*E*, *F*), and posterior pituitary (*G*, *H*) of control (*A*, *C*, *E*, *G*) and 3-day dehydrated (*B*, *D*, *F*, *H*) rats.** The IL-6 immunoreactivity was detected as described under "Experimental Procedures." A FITC fluorescent secondary antibody was used. Green cells are IL-6 immunoreactive. *Scale bars* are: 60 μm (*A–D*), 100 μm (*E*, *F*), and 200 μm (*G*, *H*).

sent detailed data for IL-6, a gene identified as being up-regulated by dehydration. To confirm and extend the array data we investigated IL-6 expression at the mRNA and protein levels throughout the HNS, both before and after an osmotic stimulus.

IL-6 mRNA Is Up-regulated by Dehydration in the SON and PVN—Using *in situ* hybridization we have shown that following 3 days of fluid deprivation of rats, IL-6 mRNA increased significantly by 3.6- ($p < 0.001$) and 2.3- ($p < 0.01$) fold, respectively, in the SON and PVN (Fig. 1). The use of a sense probe did not give a signal (data not shown). We noticed that following osmotic stimulus IL-6 mRNA level increased only in the SON and PVN and not in the control tissues hippocampus and piriform cortex where IL-6 has the same mRNA level in control and dehydrated rats. For example, the normalized optical densities of IL-6 mRNA in control and dehydrated piriform cortex were, respectively, 1.89 ± 0.32 and 1.65 ± 0.18. In the dentate gyrus of the hippocampus, the normalized optical densities of IL-6 mRNA were 2.10 ± 0.13 in control *versus* 2 ± 0.24 in dehydrated.

IL-6 Protein Level Increases by Dehydration in the SON, PVN, and Median Eminence (ME)—As mRNA levels do not necessarily reflect the final steady-state levels of the functional gene product, we used Western blotting and immunocytochem-

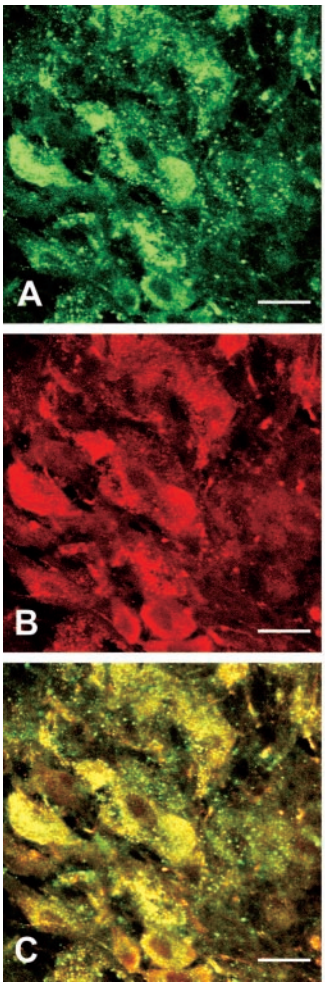

FIG. 3. **IL-6 and VP co-expression in the control (*A–C*) and 3-day dehydrated (*D–F*) rat SON.** *A* and *D*, IL-6 expression. *B* and *E*, VP expression. *C* and *F*, IL-6 and VP co-expression. Immunoreactivities were detected as described under "Experimental Procedures." FITC and Texas Red fluorescent secondary antibodies were used to reveal respectively IL-6 (*green*) and VP (*red*) immunoreactivities. *Yellow* cells are both IL-6 and VP immunoreactives. *Scale bars* are 20 μm.

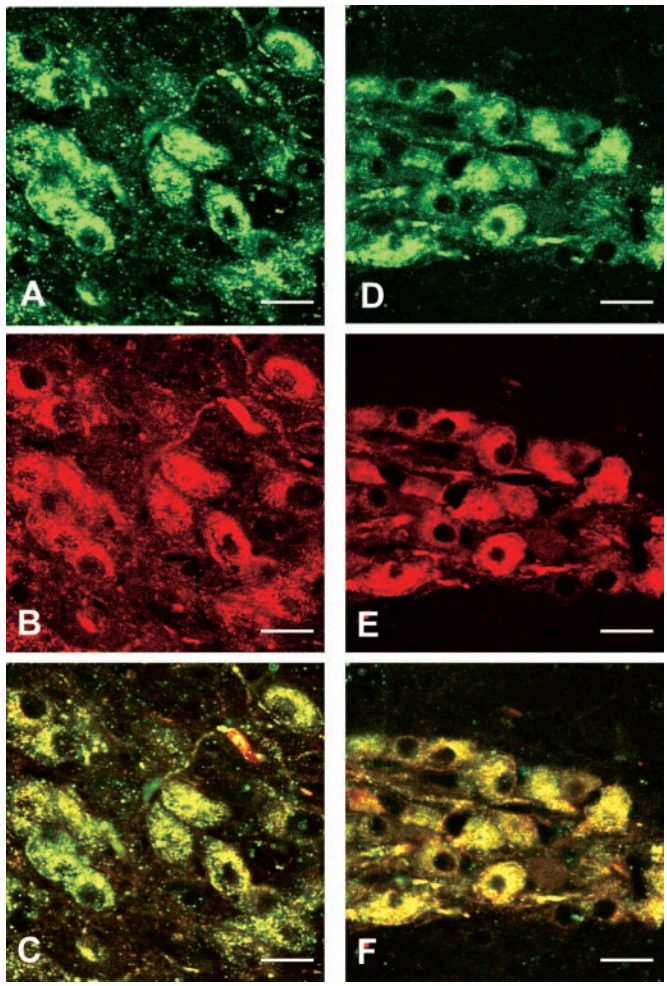

FIG. 4. **IL-6 and VP co-expression in the control (*A–C*) and dehydrated (*D–F*) rat PVN.** *A* and *D*, IL-6 expression. *B* and *E*, VP expression. *C* and *F*, IL-6 and VP co-expression. Immunoreactivities were detected as described under "Experimental Procedures." FITC and Texas Red fluorescent secondary antibodies were used to reveal respectively IL-6 (*green*) and VP (*red*) immunoreactivities. *Yellow* cells are both IL-6 and VP immunoreactives. *Scale bars* are 20 μm.

istry to assess protein levels and distribution throughout the HNS. Immunocytochemistry showed that following 3 days dehydration, IL-6-like immunoreactivity increased dramatically in the SON, PVN, and internal zone of ME (Fig. 2). Using Western blotting, we have shown 2- (4.18 ± 1.06 in control *versus* 8.53 ± 1.04 in dehydrated, $p < 0.05$) and 1.6- (4.36 ± 0.01 in control *versus* 7.07 ± 0.53 in dehydrated, $p < 0.05$) fold increases of IL-6 protein level in the SON (Fig. 6*A*) and PVN (Fig. 6*A*), respectively, following dehydration.

IL-6 Is Co-localized with VP in the SON—We then asked whether IL-6 is expressed by VP neurons of the SON and PVN. Using double staining immunocytochemistry, we showed that IL-6 is present within most VP cells in the SON (Fig. 3) and PVN (Fig. 4) of euhydrated and dehydrated rats. In addition, the same technique showed co-localization of VP and IL-6 in the internal zone of the ME (Fig. 5).

IL-6 Is Released from the Posterior Pituitary following Dehydration—IL-6 immunoreactivity in the MCN axons of the internal zone of the ME (Figs. 2 and 5) suggests that this protein can take the same secretory pathway as VP. We therefore asked whether IL-6 was present in the posterior pituitary. Using immunocytochemistry (Fig. 2) we have shown that the robust IL-6 staining seen in the posterior pituitary of euhydrated animals decreases dramatically following dehydration, suggesting a release of IL-6 from the magnocellular neuron terminals following an osmotic stimulus. This reduction was confirmed by Western blotting (2.7-fold decrease; 4.84 ± 0.61 in control *versus* 1.75 ± 0.53 in dehydrated, $p < 0.05$; Fig. 6).

IL-6 Circulating Levels do Not Increase following Dehydration—In accord with previous observations (25), serum IL-6 levels in control, euhydrated rats did not exceed the lower limit of detection of the enzyme-linked immunosorbent assay assay (>16 pg/ml). Dehydration for either 1 or 3 days had no effect on this (data not shown).

DISCUSSION

Microarray analysis is an approach for expression profiling that provides the means to perform parallel analysis of thousands of genes in a single assay (26–28). The results provide a semiquantitative assessment of whether the expression of a gene has been up- or down-regulated or remains unchanged. As such, microarrays provide a powerful tool with which to investigate biological specimens to screen for alteration in mRNA levels that accompany, and may regulate, physiological change.

Our cDNA microarray analysis revealed nine genes as being potentially differentially expressed in the SON as a consequence of dehydration (Table I). As microarray findings must be confirmed using independent methodological criteria, one of

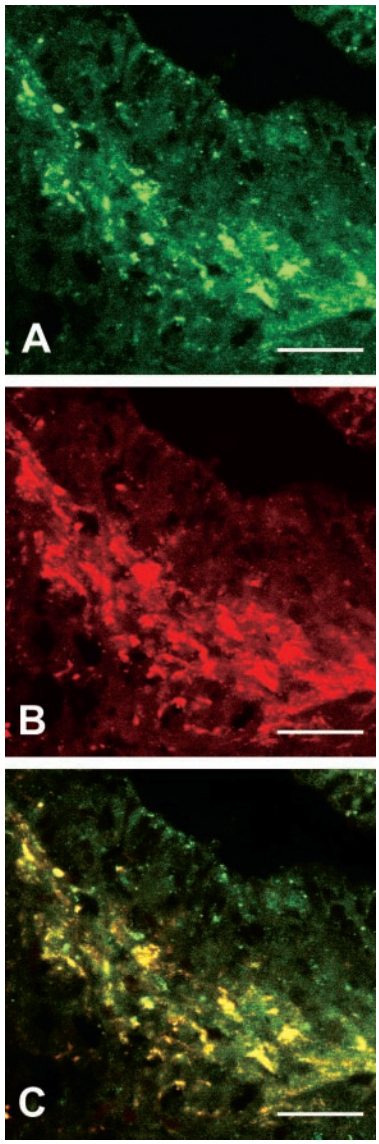

FIG. 5. **IL-6 and VP co-expression in the dehydrated rat ME.** *A*, IL-6 expression. *B*, VP expression. *C*, IL-6 and VP co-expression. Immunoreactivities were detected as described under "Experimental Procedures." FITC and Texas Red fluorescent secondary antibodies were used to reveal IL-6 (*green*) and VP (*red*) immunoreactivities. *Yellow* cells are both IL-6 and VP immunoreactivities. *Scale bars* are 80 μm.

these genes, which encodes the cytokine IL-6, was subjected to further detailed analysis.

Previous studies have shown IL-6 mRNA expression in the hippocampus, the habenuclear nucleus, the piriform cortex, the dorsomedial and ventromedial hypothalamus (29), and the rat cerebellum (30). Our ISH and immunocytochemical analyses confirmed this distribution (not shown) and also revealed robust expression in the SON and PVN (Figs. 1 and 2). IL-6 expression was found mainly, but not exclusively, in VP MCNs (Figs. 3 and 4). In further agreement with the microarray data, expression increased markedly and significantly following dehydration (Figs. 1, 2, and 6).

IL-6 immunoreactivity was also seen in the internal zone of the ME (Figs. 2 and 5) and in the posterior pituitary gland (Fig. 2). Interestingly, while dehydration provoked an increase in IL-6 expression in ME, it provoked a profound decrease in the posterior pituitary (Fig. 2), a finding confirmed by Western blotting (Fig. 6*B*). These data suggest that IL-6 takes the same secretory pathway as VP and is secreted from the posterior pituitary following dehydration.

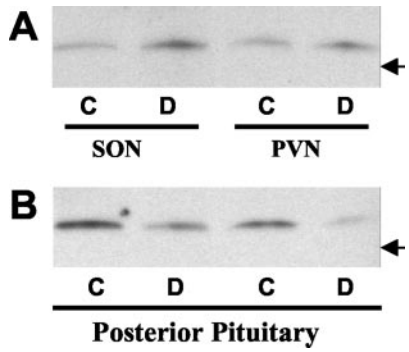

FIG. 6. **Western blots of IL-6 in the SON (*A*), PVN (*A*), and posterior pituitary (*B*).** The Western blots were performed, and IL-6-like immunoreactivity was visualized as described under "Experimental Procedures." Each lane contains 30 μg of total cell protein extracts. *Arrows* at the *right* indicate the position of a 26-kDa molecular weight standard. *C*, control; *D*, dehydrated.

Genes encoding secreted molecules co-expressed with VP are thought to have roles as autocrine or paracrine modulators of hormone release from the HNS, and the expression of some of these is regulated by osmotic stimuli. For example, peptides derived from VP and dynorphin precursors co-exist within neurosecretory vesicles of MCNs. Following an osmotic challenge, dynorphin mRNA levels increase in the SON and the PVN (31–33), but dynorphin-like immunoreactivity in the posterior pituitary is reduced following prolonged salt-loading, suggesting peptide release (34). Dynorphin, co-released with VP, is probably a local modulator of neurosecretion in the neural lobe (35, 36).

Enzyme-linked immunosorbent assay assay of serum did not reveal a measurable increase in IL-6 levels following dehydration, suggesting that the amount of IL-6 released from the posterior pituitary is insufficient to contribute significantly to the circulating pool. Instead, we suggest that IL-6, like dynorphin, might be involved in VP secretion at the level of the posterior pituitary gland. It is most likely that IL-6 exerts its effect in a paracrine fashion. A recent transgenic study showed that increased level of IL-6 following restraint stress were associated with an increase in plasma VP of GFAP-IL6 mice (37). Using a static rat hypothalamic explant incubation system, it has been demonstrated that IL-6 increases VP release (38) and that the stimulatory effect of IL-6 was blocked by cyclo-oxygenase inhibitors, suggesting the involvement of prostaglandins (38). Similarly, IL-6 increased GABA release from posterior pituitary explants, but only under depolarizing conditions (39). Again, this effect was abolished by incubation of the tissue with indomethacin, an inhibitor of cyclo-oxygenase activity, indicating that prostaglandins could mediate the stimulation of GABA release induced by IL-6 (39). Interestingly, plasma VP levels were also elevated during the 2 h after IL-6 injection in cancer patients, suggesting that IL-6 is a secretagogue of magnocellular VP secretion in humans (20). In addition, IL-6 release might also be involved in the oxytocinergic system, since it has been shown that IL-6 increases oxytocin release (38).

We have used array analysis to identify candidate genes differentially expressed in the rat SON following a 3-day dehydration stimulus. We have thus revealed IL-6 as a novel secretory product of the hypothalamo-neurohypophyseal system, the expression of which is up-regulated following dehydration. Our study has thus demonstrated the utility of array technology for the identification of new candidate genes that might be involved in HNS physiological plasticity. In this study we interrogated only 1152 gene sequences, a fraction of the total number of genes in the rat genome. Clearly our array findings

represent only a partial picture of the gene expression changes that follow dehydration. Encouraged by our results, we are currently using Affymetrix GeneChips (40) to obtain a more complete picture of the global expression patterns in control and physiologically stimulated SON.

Acknowledgment—We thank Dr. J. F. Morris (University of Oxford) for the kind gift of the anti-neurophysin-II antibody.

REFERENCES

1. Bargmann, W. (1966) *Int. Rev. Cytol.* **19,** 183–201
2. Reeves, W. B., and Andreoli, T. E. (eds) (1992) in *Posterior Pituitary and Water Metabolism. Williams Textbook of Endocrinology* (Wilson, J. D., and Foster, D. W., eds) Saunders, Philadelphia
3. Bourque, C. W., Oliet, S. H., and Richard, D. (1994) *Front. Neuroendocrinol.* **15,** 231–274
4. Honda, K., Negoro, H., Dyball, R. E., Higuchi, T., and Takano, S. (1990) *J. Physiol.* (*Lond.*) **431,** 225–241
5. Leng, G. (1982) *J. Physiol.* (*Lond.*) **326,** 35–48
6. McAllen, R. M., Pennington, G. L., and McKinley, M. J. (1990) *Am. J. Physiol.* **259,** R593–R600
7. Jhamandas, J. H., Lind, R. W., and Renaud, L. P. (1989) *Brain Res.* **487,** 52–61
8. Li, Z., and Ferguson, A. V. (1993) *Am. J. Physiol.* **265,** 302–309
9. Nissen, R., Hu, B., and Renaud, L. P. (1994) *Neuroscience* **59,** 115–120
10. Yang, C. R., Senatorov, V. V., and Renaud, L. P. (1994) *J. Physiol.* (*Lond.*) **477,** 59–74
11. Krukoff, T. L., Harris, K. H., Linetsky, E., and Jhamandas, J. H. (1994) *Neuroendocrinology* **59,** 590–602
12. Hatton, G. I. (1997) *Annu. Rev. Neurosci.* **20,** 375–397
13. Murphy, D., Si-Hoe, S. L., Brenner, S., and Venkatesh, B. (1998) *Bioessays* **20,** 741–749
14. Burbach, J. P., Luckman, S. M., Murphy, D., and Gainer, H. (2001) *Physiol. Rev.* **81,** 1197–1267
15. Theodosis, D. T., El Majdoubi, M., Pierre, K., and Poulain, D. A. (1998) *Cell. Mol. Neurobiol.* **18,** 285–298
16. Tanaka, M., Cummins, T. R., Ishikawa, K., Black, J. A., Ibata, Y., and Waxman, S. G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1088–1093
17. Eisen, M. B., and Brown, P. O. (1999) *Methods Enzymol.* **303,** 179–205
18. Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995) *Science* **270,** 467–470
19. Duggan, D. J., Bittner, M., Chen, Y., Meltzer, P., and Trent, J. M. (1999) *Nat. Genet.* **21,** 1 suppl., 10–14
20. Mastorakos, G., Weber, J. S., Magiakou, M. A., Gunn, H., and Chrousos, G. P. (1994) *J. Clin. Endocrinol. Metab.* **79,** 934–939
21. Mastorakos, G., Magiakou, M. A., and Chrousos, G. P. (1995) *Ann. N. Y. Acad. Sci.* **771,** 438–448
22. Gionis, D., Ilias, I., Moustaki, M., Mantzos, E., Papadatos, I., Koutras, D. A., and Mastorakos, G. (2003) *J Pediatr Endocrinol. Metab.* **16,** 49–54
23. Vawter, M. P., Barrett, T., Cheadle, C., Sokolov, B. P., Wood, W. H., III, Donovan, D. M., Webster, M., Freed, W. J., and Becker, K. G. (2001) *Brain Res. Bull.* **55,** 641–650
24. Zeng, Q., Carter, D. A., and Murphy, D. (1994) *J. Neuroendocrinol.* **6,** 469–477
25. Cartmell, T., Poole, S., Turnbull, A. V., Rothwell, N. J., and Luheshi, G. N. (2000) *J. Physiol.* (*Lond.*) **526,** 653–661
26. Young, R. A. (2000) *Cell* **102,** 9–15
27. King, H. C., and Sinha, A. A. (2001) *J. Am. Med. Assoc.* **286,** 2280–2288
28. Murphy, D. (2003) *Adv. Physiol. Edu.* **26,** 256–270
29. Schobitz, B., de Kloet, E. R., Sutanto, W., and Holsboer, F. (1993) *Eur. J. Neurosci.* **5,** 1426–1435
30. Gadient, R. A., and Otten, U. (1994) *Neurosci. Lett.* **182,** 243–246
31. Sherman, T. G., Civelli, O., Douglass, J., Herbert, E., and Watson, S. J. (1986) *Neuroendocrinology* **44,** 222–228
32. Sherman, T. G., Civelli, O., Douglass, J., Herbert, E., Burke, S., and Watson, S. J. (1986) *Fed. Proc.* **45,** 2323–2327
33. Lightman, S. L., and Young, W. S., 3rd. (1987) *J. Physiol.* (*Lond.*) **394,** 23–39
34. Meister, B., Cortes, R., Villar, M. J., Schalling, M., and Hokfelt, T. (1990) *Cell Tissue Res.* **260,** 279–297
35. Schafer, M. K., and Martin, R. (1994) *Biol. Chem. Hoppe-Seyler* **375,** 737–740
36. Russell, J. A., Leng, G., and Bicknell, R. J. (1995) *Exp. Physiol.* **80,** 307–340
37. Raber, J., O'Shea, R. D., Bloom, F. E., and Campbell, I. L. (1997) *J. Neurosci.* **17,** 9473–9480
38. Yasin, S. A., Costa, A., Forsling, M. L., and Grossman, A. (1994) *J. Neuroendocrinol.* **6,** 179–184
39. De Laurentiis, A., Pisera, D., Lasaga, M., Diaz, M., Theas, S., Duvilanski, B., and Seilicovich, A. (2000) *Neuroimmunomodulation* **7,** 77–83
40. Lipshutz, R. J., Fodor, S. P., Gingeras, T. R., and Lockhart, D. J. (1999) *Nat. Genet.* **21,** Suppl. 1, 20–24